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ORIGINAL ARTICLE

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Abstract Expression pattern and immunogenicity are critical issues that define tumor antigens as diagnostic markers and potential targets for immunotherapy. The development of SEREX (serological analysis of recombinant expression libraries) has provided substantial progress in the identification of tumor antigens eliciting both cellular and humoral immune responses in cancer patients. By SEREX, we have previously identified RAB38/NY-MEL-1 as a melanocyte differentiation antigen that is highly expressed in normal melanocytes and melanoma tissues but not in other normal tissues or cancer types. In this study, we further demonstrate that RAB38/NY-MEL-1 is strongly immunogenic, leading to spontaneous antibody responses in a significant proportion of melanoma patients. The immune response occurs solely in malignant melanoma patients and was not detected in patients with other diseases, such as vitiligo, affecting melanocytes. Fine analysis of the spontaneous anti-RAB38/NY-MEL-1 antibody response reveals a polyclonal B cell recognition targeting various epitopes, although a dominant immunogenic region was preferentially recognized. Interestingly, our data indicate that this recognition is not rigid in the course of a patient's response, as the dominant epitope changes during the disease evolution. Implications for the understanding of spontaneous humoral immune responses are discussed.

Keywords RAB38/NY-MEL-1 · Tumor antigen · Humoral immune response · SEREX · B cell epitope · Malignant melanoma

Alfred Zippelius and Asma Gati contributed equally to this work.

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Introduction

Humoral immune responses directed against tumor antigens are frequently observed in cancer patients, reflecting the interaction between the immune system and developing tumors. The properties of self-antigens necessary to induce immune responses specific to cancer are still poorly defined. Genetic aberrations [4, 27, 29], changes in the level of gene expression [22, 30], or aberrant expression in tumor lesions [23, 26] have been shown as potential mechanisms for immunogenicity, and serological responses against these antigens have been reported in cancer patients. In the last decade, the development of novel immunological methods has greatly contributed to our understanding of serological autologous immune responses in tumor patients (for a review, see [10]). SEREX (serological analysis of recombinant expression libraries), in particular, an expression cloning strategy, has led to the definition of a large repertoire of immunogenic antigens in various tumor types [24]. Ideally, this technique distinguishes antigens with a direct relevance to cancer etiology or cancer progression from antigens that represent general autoimmunogenic cellular components and provides attractive antigenic targets for immunotherapy approaches in cancer patients.

Among the SEREX-defined repertoire of tumor antigens, cancer-testis (CT) antigens and differentiation antigens have been of particular interest with respect to immunotherapy strategies in cancer [28]. The first category of antigens (e.g. NY-ESO-1, MAGE, SSX-2, etc.) are expressed in normal adult tissues solely in testis, but are present/activated in a variety of different tumor types in a lineage-unrestricted fashion. In contrast, differentiation antigens, e.g. Melan-A/MART1, tyrosinase, and gp100, are expressed in tissues and tumors of a particular cell lineage such as melanocytes. Melanocyte differentiation antigens frequently induce specific serological and T cell responses in melanoma patients [5, 7, 12, 32].

In a previous SEREX screening of a melanoma cell line library, we identified RAB38/NY-MEL-1 (later RAB38) [9], a polypeptide of 211 amino acids resembling a new member of the rab family of G proteins (for a review, see [21]). Preliminary data indicate a highly regulated expression that is restricted to the melanocytic cell lineage [9]. Similar to most other melanosomal proteins, a coat color defect mapping to the RAB38 genetic locus has recently been identified in the mouse system [16]. In addition to five highly conserved regions necessary for GTP binding, the structural features are characterized by a unique COOH terminus which allows post-translational lipid modifications. Interestingly, intracellular localization of a rat homolog with 97% amino acid identity indicates that RAB38 is expressed extensively in the cytoplasm with a distribution pattern similar to the endoplasmic reticulum [18, 19].

In this study, we further analyzed the expression pattern and immunogenicity of RAB38. As anticipated, RAB38 exhibits the characteristics of a melanocyte differentiation antigen as it is abundantly expressed in normal melanocytes and melanoma and not in any other normal tissues or non-melanocytic tumor. Spontaneous humoral immune responses against RAB38 are characterized by (i) their presence in a large proportion of melanoma patients, (ii) their restriction to patients with melanoma but not other tumor types, and (iii) polyclonal responses targeting various epitopes with a dominant immunogenic region identified. The restricted expression pattern and the immunogenicity of this antigen suggest that RAB38 may be used as a marker protein and as an appropriate target for antigen-specific immunotherapy in patients with malignant melanoma.

Materials and methods

Sera, tumor samples, and melanoma cell lines

Twelve metastatic melanoma lesions derived from 10 melanoma patients, and serum samples from 52 melanoma patients, 15 non-melanoma cancer patients, 13 vitiligo patients, and 15 healthy individuals were obtained at the University Hospital, Zurich, Switzerland, and Krankenhaus Nordwest Frankfurt, Germany, under consideration of local legal regulations. Melanoma cell lines were derived from the repository of the Ludwig Institute for Cancer Research, New York Branch at Memorial Sloan-Kettering Cancer Center.

RNA extraction and real-time RT-PCR

Total RNA was extracted from melanoma lesions by the Qiagen RNA Extraction Kit (Qiagen, Hombrechtikon, Switzerland) and quantified by spectroscopy (SmartSpec 3000 Spectrophotometer, BioRad). In addition, RNA was obtained from 16 different normal tissues (Clontech, Basel, Switzerland) and cultured human melanocytes (Gentaur). RNA was reverse-transcribed into cDNA using the TaqMan EZ RT-PCR kit (Applied Biosystems, Rotkreuz, Switzerland). Gene-specific TaqMan probes with a predetermined, optimum concentration of gene-specific forward and reverse primers (300–900 nM) were purchased

from Applied Biosystems (TaqMan Gene Expression Assay on Demand). For endogenous control, 18S RNA-specific primers/probes were purchased from Applied Biosystems. The PCR consisted of 40 cycles of 95°C denaturation (15 s) and of 60°C annealing/extension (60 s). A total of 20 ng/μl were reverse-transcribed and 2.5 μl of cDNA was diluted in TaqMan Universal PCR Master Mix supplemented with (Fam)-labeled gene-specific TaqMan probe, and an optimal concentration of the gene-specific forward and reverse primers (300–900 nM) were used for PCR. All PCR reactions were run as triplicates, and thermal cycling and fluorescent monitoring were performed using an ABI7000 thermal cycler (Applied Biosystems).

Production of the recombinant RAB38 protein

The entire coding region of RAB38 (636 bp) was amplified with primers including specific cleavage sites for *Bam*H1 (*Bam*-5′: CACACAGGATCCATGCA GGCCCCGCACAAGGAG) and *Hin*dIII (*Hin*d-3′: C ACACAAAGCTTCTAGGATTTGGCACAGCCA GAG). After digestion with *Bam*H1 and *Hin*dIII, the PCR product was cloned into the pQE30 expression vector (Qiagen). Competent M15 (pREP4) *Escherichia coli* were transformed and the recombinant protein was produced and purified under denaturing condition following the manufacturer's protocol. The purified recombinant His-tag protein was analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), followed by silver staining.

Detection of RAB38-specific antibody responses

For Western blot assays, the purified recombinant protein and lysate of a RAB38-expressing melanoma cell line (SK-MEL-37) resuspended in 1:2 in Laemmli sample buffer and denaturated for 5 min at 95°C were used. 1D SDS–PAGE was performed on 15% acrylamide gel. The protein was then transferred onto Protan Nitrocellulose Membranes (Schleicher and Schuell, BioScience, Dassel, Germany) in a Mini Trans-Blot cell (BioRad). The efficiency of the electrotransfer was assessed by Ponceau Red staining of the membranes. After incubation with patients' sera diluted at 1:250 for 60 min, reactivity was determined using an AP-conjugated goat-anti-human IgG-specific antibody (Jackson Immuno Research Laboratories, LaRoche, Switzerland) diluted 1:5,000. Immunoreactive proteins were visualized with BCIP and NBT (Roche, Rotkreuz, Switzerland). All steps were performed at room temperature.

For ELISA assays, 96-well plates (Corning, Wohlen, Switzerland) were coated with 50 ng of recombinant RAB38 protein, tetanus toxoid (Berna), or overlapping peptides of 18 aa length derived from the RAB38 protein sequence (BioSynthesis, Lewisville, TX, USA). All peptides were diluted in PBST (PBS/0.5% Tween-20). Plates were incubated overnight at 4°C and consecutively blocked with PBST/5% fetal calf serum (FCS) for 2 h at 4°C and then washed twice. Serum samples diluted 1:250 in PBST/5% FCS were incubated for 2 h at room temperature, followed by incubation for 30 min with a secondary, peroxidase-conjugated, goat-anti-human IgG antibody (Sigma, Darmstadt, Germany), diluted 1:20,000 in PBST/5% FCS. The plates were subsequently developed at room temperature for 30 min with 150 μl/well tetramethylbenzidine (Sigma, Darmstadt, Germany) and analyzed using an ELISA reader (Labexim LMR1) at $\lambda = 450$ nm.

Immunohistochemistry

Tissues were mounted in OCT, snap-frozen in isopentane pre-cooled liquid nitrogen upon surgical removal. Five micrometers thick frozen tissue sections were placed on glass slides, air-dried, fixed with cold acetone for 10 min, and stored at −70°C. As a positive control, melanoma cell line SK-MEL-37 grown in slide chambers was used. After removal of media, cells were air-dried and fixed with cold acetone (data not shown). Slides were incubated with affinity-purified polyclonal rabbit anti-RAB38 antibodies [18, 19], diluted 1:200 in TBS for 30 min at room temperature. Primary antibodies were detected with a goat anti-rabbit secondary, followed by alkaline phosphatase labeled donkey antibodies to goat immunoglobulins (Jackson ImmunoResearch Laboratories). Dilutions of secondary reagents were made in TBS containing 1% FCS. Alkaline phosphatase was then detected by a red color reaction using naphthol AS-BI phosphate as a substrate and new fuchsin as a chromogen. Endogenous alkaline phosphatase was blocked by levamisole. Slides were counterstained with Meyer's hematoxylin for 2 min.

Prediction of the protein structure and B cell epitopes

Hydrophilicity was calculated based on the Kyte–Doolittle [14] method with a window size of 17 amino acid residues. Surface accessibility and buried residues were calculated using the ExpASy (Expert Protein Analysis System) with a window of 17 amino acid residues [20]. To visualize potential binding sites depending on the 3D structure, the protein sequence was blasted against the pdb database [1]. 3D graphs were designed using the RasMol software Version 2.6.

Results

RAB38 mRNA expression in normal tissues and melanomas

Extending our previously reported data [9], we quantified mRNA expression in a panel of different normal tissues and melanoma lesions by real-time RT-PCR. The analysis of normal tissues is shown in Fig. 1a. Relative to the mRNA level in normal melanocytes, which was arbitrarily set as 100%, the adrenal gland showed a weak expression of RAB38 mRNA (4%), whereas all other normal tissues revealed a marginal (≤1%) to absent RAB38 mRNA expression. In contrast, 10 out of 12 metastatic melanoma lesions expressed RAB38 transcripts (Fig. 1b). In particular, four lesions (ZH-104, ZH-23a, ZH-174, and ZH-122a) showed strong overexpression of RAB38 mRNA up to 22-fold compared to that of melanocytic expression; five melanoma lesions (ZH-23b, ZH-122b, ZH-132, ZH-167, and ZH-274) had RAB38 mRNA levels similar to melanocytes. Two metastatic lesions (ZH-346 and ZH-256) showed no significant RAB38 mRNA expression and were derived from an amelanotic melanoma and an uveal melanoma. In agreement with the common phenomenon of heterogenous tumor antigen expression, we noticed variable expression levels of RAB38 mRNA in different metastases derived from the same patient (ZH-23a vs. ZH-23b and ZH-122a vs. ZH-122b).

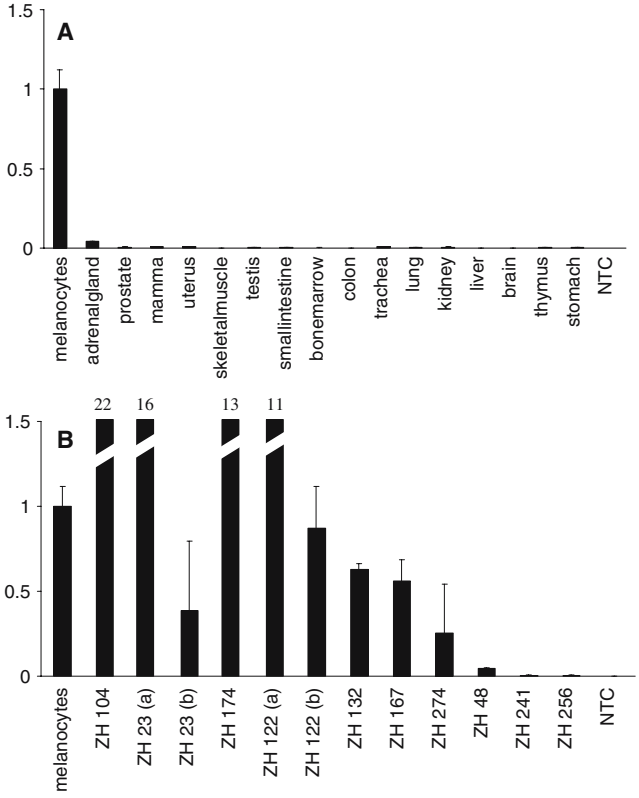

Fig. 1 RAB38 cDNA-specific real-time *RT-PCR* analysis of 16 normal tissues (**a**) and 12 melanoma lesions obtained from patients with malignant melanoma (**b**). The data are expressed relative to the mRNA level in normal melanocytes, arbitrarily set as 100%. The value of each sample was determined in triplicate reactions. *NTC* no template control

RAB38 protein expression in malignant melanoma

Owing to a high homology to the rat rab-related protein, sharing 97% amino acid identity, an affinity-purified rabbit anti-rat polyclonal RAB38 antibody [18, 19] was used to stain human melanoma lesions. Immunostaining of melanoma cell line SK-MEL-37 (Fig. 2a) and melanoma lesions (ZH-122b and ZH-23b; Fig. 2b, c, respectively) showed cytoplasmic immunoreactivity in RAB38-positive melanoma cells. Level of RAB38 transcripts, as determined by real-time PCR, correlated with the immunostaining results: ZH-122b, which showed RAB38 mRNA level of about 90% compared to human melanocytes, revealed homogeneous immunostaining, whereas ZH-23b (expression level of 40% of melanocytes) showed more restricted immunopositivity. No staining was present in melanoma ZH-241 that was RAB38-negative by real-time RT-PCR (Fig. 2d). No staining was present in surrounding non-melanocytic tissue components such as stroma, keratinocytes, smooth muscle cells, connective tissue cells, and endothelial cells (Fig. 2b).

Melanoma patients frequently develop humoral immune responses against RAB38

RAB38 was previously identified in a SEREX analysis of an autologous melanoma cell line library [9]. To further assess the frequency and specificity of anti-RAB38 humoral responses, we screened 95 sera derived from 52 melanoma patients, 15 non-melanoma cancer patients, 13 vitiligo patients, and 15 healthy individuals for anti-RAB38 antibodies by ELISA. Sera from healthy individuals allowed the definition of a cut-off value (0.271), defined as the mean OD plus 3× standard deviations (0.22±3×0.017). To assess the general immune competence of the individuals analyzed, vaccine-induced humoral immune responses against a recombinant tetanus toxoid protein were tested. The frequency of tetanus-toxoid-specific antibody responses was not significantly different in melanoma patients vs. non-melanoma cancer patients or non-cancer patients, respectively (61% vs. 87 or 87%; $p > 0.05$, Fig. 3a). This frequency is also well compatible

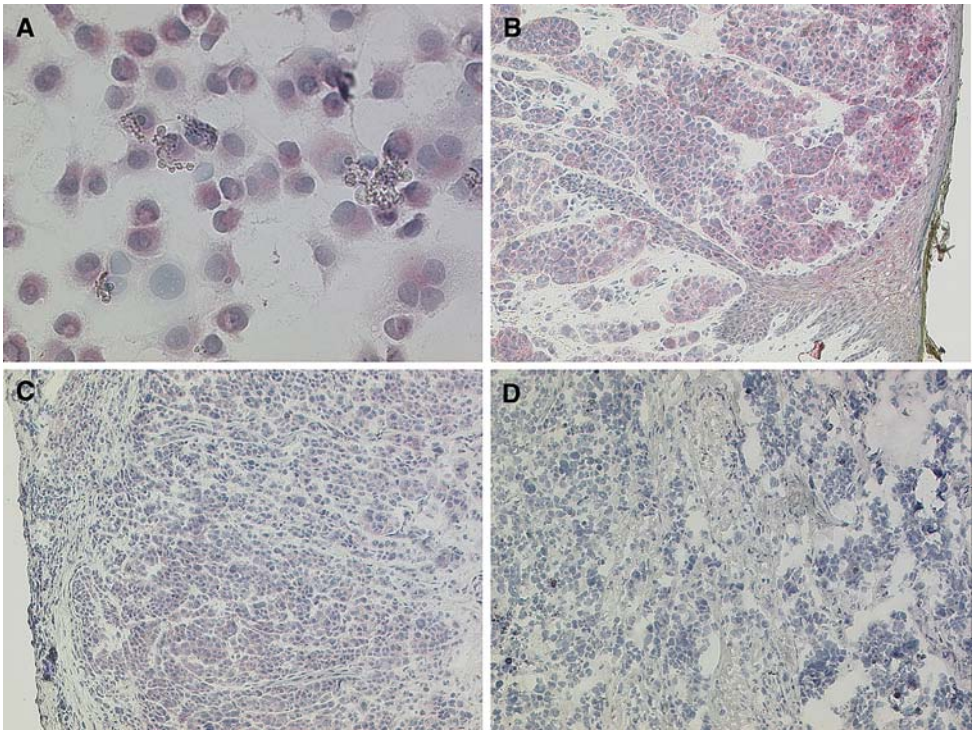

Fig. 2 Immunohistochemical staining of a melanoma cell line (SK-MEL-37, **a**) and three melanoma lesions (ZH-122b, **b**; ZH-23b, **c**; and ZH-241, **d**) using a polyclonal RAB38 antibody [18, 19]. Magnification: ×40 (**a**–**d**)

with previously reported data [25]. In contrast, anti-RAB38 antibodies were detected exclusively in sera derived from melanoma patients. All non-melanoma cancer patients, vitiligo patients, and healthy individuals were tested negative. RAB38-specific antibodies were detected in 12 out of 52 sera (23%) derived from melanoma patients (Fig. 3b), with titers ranging from 1:100 to 1:1,000 (data not shown). Some positive sera in ELISA assays were confirmed by Western blotting (data not shown). Specificity of these antibodies was demonstrated by testing antibody-positive sera against the recombinant RAB38 protein as well as a RAB38-positive melanoma cell line lysate (SK-MEL-37) by Western blotting (Fig. 3c, d). A protein species migrating at approximately 23 kDa was recognized in both lanes, confirming the specific immunoreactivity.

Identification of RAB38 epitopes recognized by antibodies present in melanoma sera

To further explore antibody epitopes and immunogenicity of antigenic sites within the RAB38 protein, reactivity of patients' sera was tested against 25 overlapping peptides of 18 amino acid residues that span the entire RAB38 protein sequence. Background reactivity against the peptides was determined using the sera from three healthy individuals, calculated as mean OD plus 3 × standard deviations ($OD_{control}$). Samples with OD values ≥2-fold above the background were considered positive. Sera derived from seven RAB38 antibody-positive melanoma patients (NW-2231, ZH-48, NW-37, NW-1274, MZ-19-MEL, ZH-104, and NW-1751) were used for epitope mapping and the results were shown in Fig. 4. Sera from melanoma patients showed significant reactivity with distinct peptides within the RAB38 protein sequence. In particular, reactivity against the amino terminal part (aa 41–58) and the central part (aa 73–114) was observed in three of the seven patients (ZH-48, ZH-104, and NW-2231) and four of the seven patients (ZH-48, NW-37, NW-1751, and NW-2231), respectively. All patients' sera showed reactivity against peptide sequences in the carboxy terminus. Notably, all seven sera recognized at least peptide 20 and/or peptide 21, indicating that the represented segment (aa 153–178) is preferentially antigenic. However, reactivity was not restricted to those sites, as other regions were also recognized, but at lower frequencies. To confirm the potential immunogenicity of the RAB38 region represented by peptides 20 and 21 (aa 153–178), we performed a computer prediction analysis based on hydrophilic profiles using the Kyte–Doolittle method [14]. We identified several hydrophilic regions within the amino acid sequence of RAB38 (Fig. 5a), which tend to localize on the surface of the molecule. Additionally, applying the ExpASy program, we were able to predict the sequences exposed on the surface of the RAB38 protein (Fig. 5b) as well as the sequences buried to the interior of the

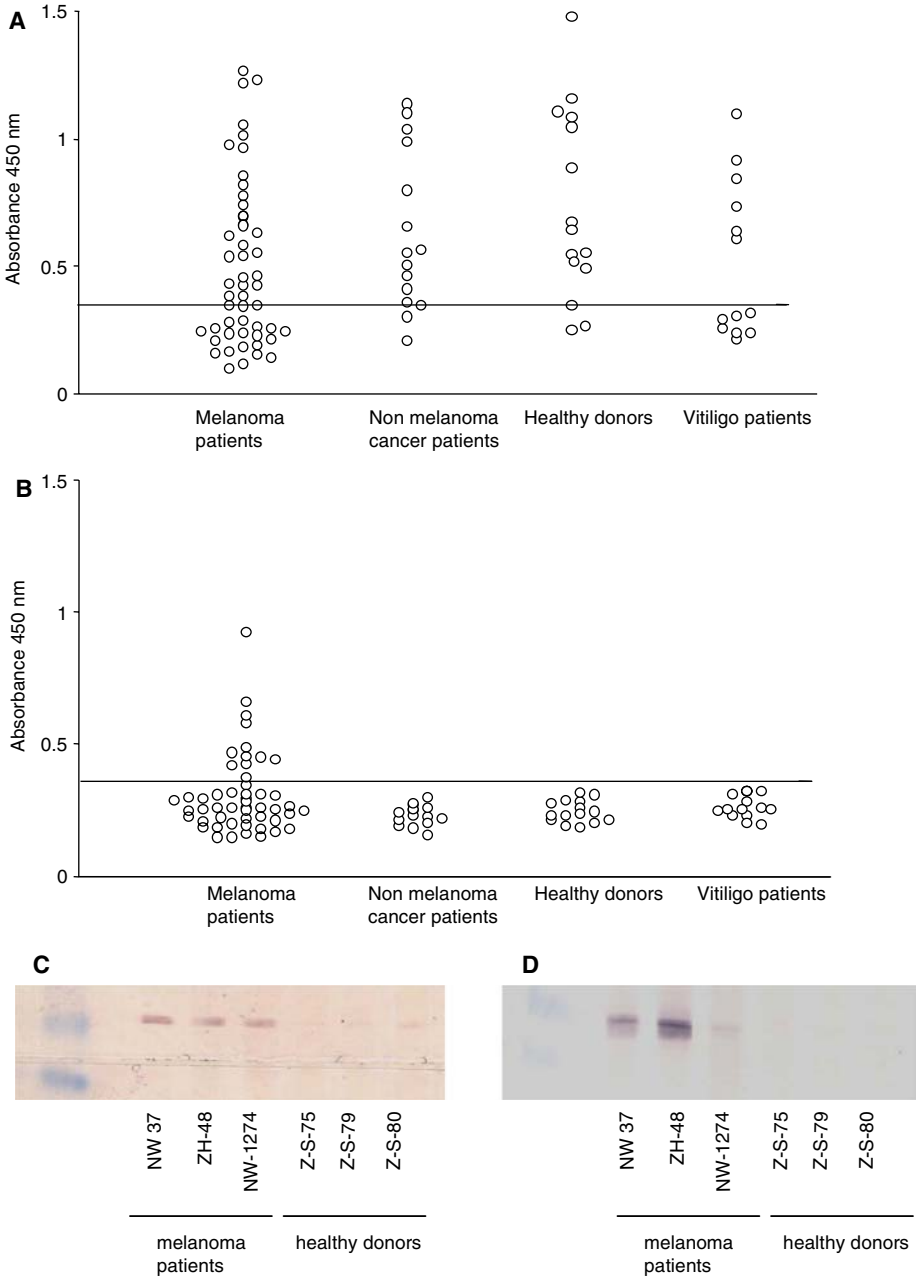

Fig. 3 Tetanus-toxoid-specific (**a**) and RAB38-specific antibody responses (**b**) in sera from melanoma patients, non-melanoma cancer patients, healthy donors, and vitiligo patients evaluated in ELISA assays using the recombinant RAB38 and tetanus toxoid protein, respectively. The *dashed line* indicates a cut-off value (i.e. mean OD ± 3 × standard deviations), as determined in ELISA assays with the recombinant RAB38 protein using sera from healthy individuals. To determine the specificity of RAB38 antibody-positive sera from melanoma patients, the recombinant RAB38 protein (**c**) and lysate of a RAB38-positive melanoma cell line (SK-MEL-37, **d**) were transferred onto nitrocellulose membranes and probed with sera from RAB38 antibody-positive melanoma patients (NW-37, ZH-48, and NW-1274) and healthy donors (Z-S-75, Z-S-79, and Z-S-80)

protein (Fig. 5c). Importantly, only the region aa 153–178 recognized by all seven patients' sera was predicted to be both accessible and hydrophilic. Figure 5d visualizes the preferential antigenic region on a 3D structure of the RAB38 protein. To assess the epitope profile of individual patients over the course of the disease, follow-up sera from two seropositive patients were tested. Figure 6 shows a stable antibody titer against the RAB38 protein over 3 years for patient NW-37 and 7 months for patient NW-1274 (Fig. 6a, b). Although the antibody titer against the recombinant protein remains stable, a switch of the predominant epitope was observed for patient NW-1274 (Fig. 6c). In comparison, the epitope profile for patient NW-37 remained stable over more than 3 years (Fig. 6d).

Discussion

The recognition of tumor cells by the immune system is reflected by spontaneous cellular and humoral immune responses. The immune system can target and destroy tumor cells via the recognition of specific tumor antigens [3]. Promising target antigens for immunotherapeutic approaches are tumor-restricted immunogenic proteins that are expressed at high levels in malignant

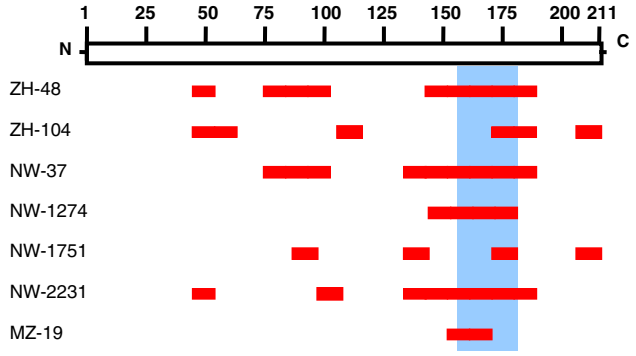

Fig. 4 Detection of antibodies against single RAB38-derived peptides. Sera from melanoma patients with humoral immune responses against RAB38 (NW-2231, ZH-48, NW-1274, MZ-10-MEL, ZH-104, and NW-1751) were tested in ELISA assays using overlapping peptides that span the entire RAB38 protein sequence (see Materials and methods). Background reactivity against the peptides (i.e. mean OD $\pm$ 3 $\times$ standard deviations) was determined in ELISA assays using sera from healthy individuals. Samples with OD values $\geq$twofold above the background were considered positive

cells. A growing number of tumor antigens have been identified by methods based on autologous T cell responses and serological responses in cancer patients. To date, only few of these antigens, e.g. NY-ESO-1, have been reported to frequently elicit spontaneous cellular as well as humoral immune responses. Like RAB38, the CT antigen NY-ESO-1 was initially identified based on a spontaneous antibody response employing the SEREX method [2]. Interestingly, most NY-ESO-1 seropositive patients have simultaneous NY-ESO-1-specific CD8 and CD4 T cell responses [6, 11]. This observation supports the strategy of exploiting serological responses in order to identify relevant T cell epitopes that can be used as targets for vaccine-based immunotherapy. Ideal target antigens should exhibit a frequent and tumor-restricted expression as well as a high immunogenicity, as reflected by frequent spontaneous immune responses in patients with antigen-positive tumors.

Here we report on RAB38, a novel melanocyte differentiation antigen, that frequently induces humoral immune responses in melanoma patients. Extending the initial expression analysis by northern blot [9], we performed real-time RT-PCR in normal tissues and melanoma lesions, and confirmed the tissue-restricted expression of RAB38 with predominant expression in melanocytes and in melanoma tissues. The low-level mRNA expression in adrenal gland needs to be confirmed in a higher number of cases and especially

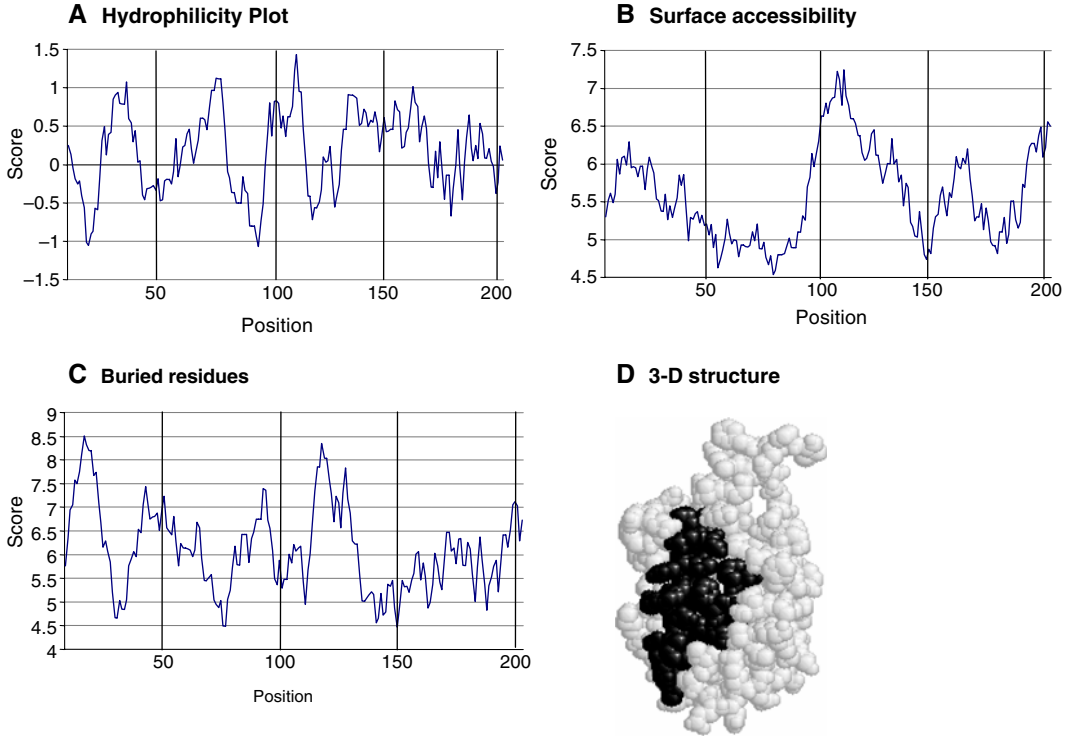

Fig. 5 Computer-aided prediction of B cell epitopes from RAB38. **a** The hydrophilicity score of the RAB38 protein was calculated with Kyte–Doolittle method [14]. Using a window size of 17 amino acid residues, the RAB38 protein was predicted for surface accessibility (**b**) and buried residues (**c**). Localization of the immunodominant region (aa 153–178) within the predicted 3D structure of the RAB38 protein (**d**)

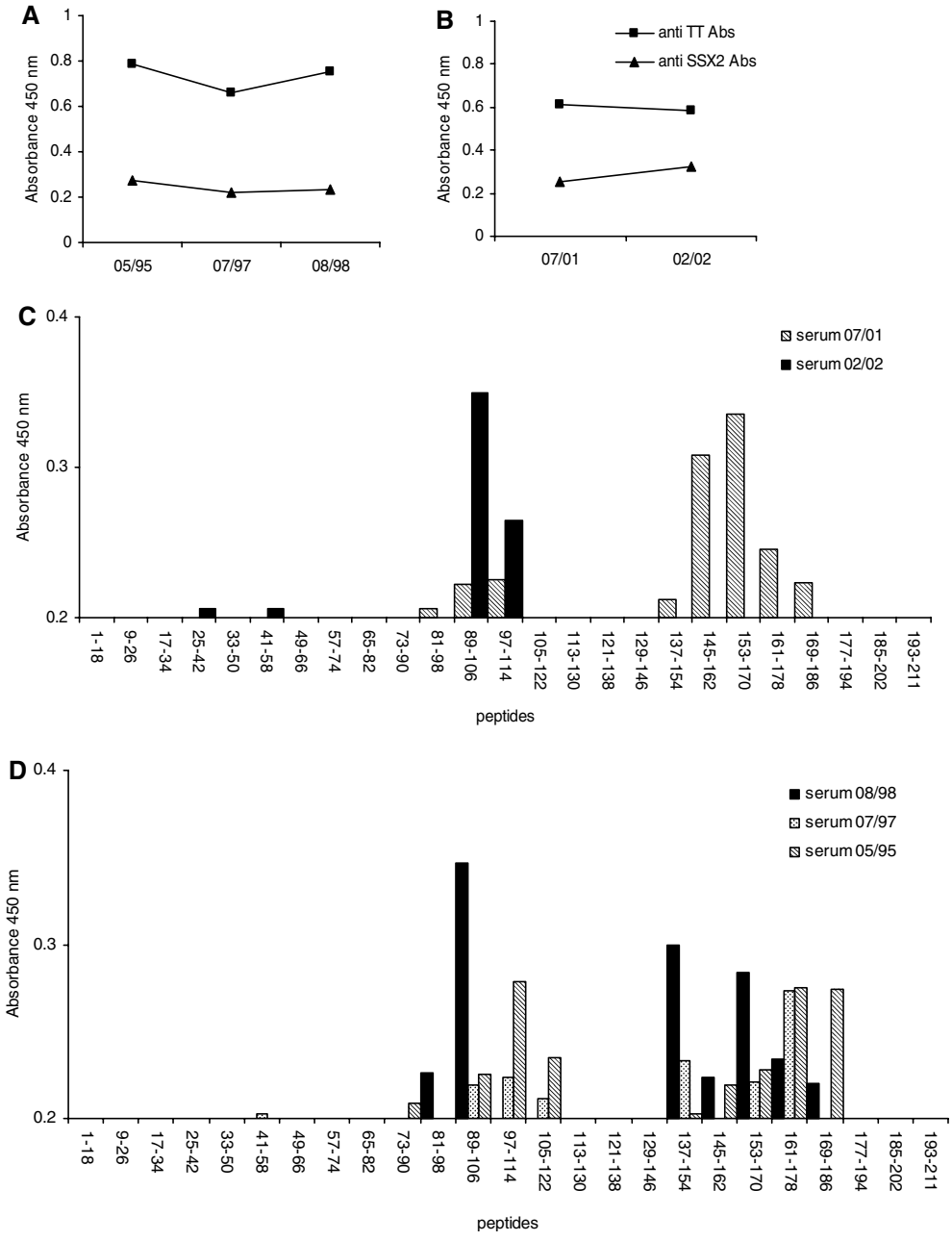

Fig. 6 RAB38-specific antibody responses in individual patients over the course of the disease. Sera of two melanoma patients NW-37 (**a**, **c**) and NW-1274 (**b**, **d**) were tested in ELISA assays using the recombinant RAB38 protein (**a**, **b**) and overlapping peptides as mentioned above (**c**, **d**)

on a protein level. However, like melanocytes, adrenal medulla is a neuroectodermal tissue and some relation between both tissues may exist. Among the melanomas of our series, the only negative cases were specimens of an amelanotic melanoma and uveal melanoma. Amelanotic melanoma is characterized by the low number of melanosomes which may explain the low expression of some melanocyte differentiation antigens. However, on a protein level, melanocyte differentiation antigens such as Melan-A, gp100, and tyrosinase did not show a lower expression in amelanotic melanoma. The biology of uveal melanoma is poorly understood and it has been reported that the expression of melanocyte lineage markers differs from cutaneous melanoma [8] (E. Jaeger, personal communication). RAB38 mRNA expression data obtained by real-time RT-PCR correlated to RAB38 protein expression analyzed by immunohistochemistry using a polyclonal rabbit anti-rat RAB38 antibody. RAB38-positive cells showed cytoplasmatic staining

which is in line with previous reports localizing murine RAB38 to the melanosomal compartment in mice [16]. We are currently generating mAbs to human RAB38 in order to analyze its protein expression in normal tissues and tumors.

In addition to its tumor-restricted expression, we demonstrated that RAB38 is highly immunogenic in melanoma patients. Spontaneous RAB38-specific antibody responses were present in 23% of melanoma patients (12 out of 52). As not all melanomas express RAB38, the frequency of anti-RAB38 response in patients with RAB38-positive melanoma is likely to be higher. The immune response was present solely in melanoma patients and none of the 13 vitiligo patients had detectable RAB38-specific antibodies. This suggests that serological responses to RAB38 likely result from ongoing interactions of the immune system with evolving and progressing tumors, rather than a tumor-unrestricted process following cell necrosis and release of intracellular proteins. However, this does not exclude the possibility of side effects such as vitiligo as a result of immune responses to RAB38 vaccines as it has been observed in clinical trials using vaccines to other melanocyte differentiation antigens such as Melan-A and tyrosinase [13, 17].

To identify antibody epitopes within the RAB38 sequence, we tested RAB38 antibody-positive sera against RAB38-derived peptides in ELISA assays and correlated the serological findings with the results of computer algorithms predicting potential immunogenic sites in the protein structure [15, 20]. The results of both methods concurred indicating an immunodominant region encompassing position aa 153–178. Notably, this dominant B cell epitope may by used to measure antibody responses, as previously reported for NY-ESO-1 [31]. This would bypass the requirement for purification of the recombinant protein which is often difficult to achieve. However, as demonstrated here, a potential caveat of the peptide-based approach for measuring antibodies is the possibility of changing the epitope during the course of the disease (Fig. 6c). Consequently, changing epitopes may simply be missed in epitope-restricted assays. Moreover, the present approach is not suitable for the identification of conformational epitopes.

In conclusion, the present data support our initial analyses and demonstrate that RAB38 is a true melanocyte differentiation antigen which may be useful for diagnostic purposes or may serve as a target for immunotherapy of melanoma. Further studies about the protein expression and potential T cell responses are necessary and underway to assess the role of RAB38 as a cancer target and diagnostic marker.

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